

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
21 October 2004 (21.10.2004)

PCT

(10) International Publication Number
WO 2004/090167 A1

(51) International Patent Classification⁷: C12Q 1/68, 1/66

(21) International Application Number:
PCT/SG2004/000093

(22) International Filing Date: 14 April 2004 (14.04.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/462,308 14 April 2003 (14.04.2003) US

(71) Applicant (for all designated States except US):
TEMASEK LIFE SCIENCES LABORATORY
[SG/SG]; 1 Research Link, National University of Singa-
pore, Singapore 117604 (SG).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **HONG, Yan** [SG/SG];
Block 206, Bukit Batok Street 21, #06-80, Singapore
650206 (SG).

(74) Agent: **ELLA CHEONG SPRUSON & FERGUSON**
(SINGAPORE) PTE LTD; P.O. Box 1531, Robinson
Road Post Office, Singapore 903031 (SG).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Euro-
pean (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR,
GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: DETECTION OF A TARGET NUCLEIC ACID, BY POLYMERASE REACTION AND ENZYMATIC DETECTION
OF RELEASED PYROPHOSPHATE

(57) Abstract: The present invention provides methods of identifying the presence of a target nucleic acid in a sample wherein the
target nucleic acid is replicated and said replication of the target is detected as the consumption of a deoxynucleotide triphosphate
precursors whereby any release of PPI is indicative of incorporation of deoxynucleotide or dideoxynucleotide and thus replication of
the target DNA. The invention also provides kits and reaction mixtures utilized in the methods of the invention.

WO 2004/090167 A1

Best Available Copy

**DETECTION OF A TARGET NUCLEIC ACID, BY POLYMERASE REACTION
AND ENZYMATIC DETECTION OF RELEASED PYROPHOSPHATE**

5 The present invention is directed to methods for the detection and/or quantification of a nucleic acid in a sample. More specifically, the invention is directed to detecting a nucleic acid in a sample by using the nucleic acid as a template for nucleic acid replication and detecting the release of pyrophosphate (PPi) during the replication process.

10 The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference.

BACKGROUND OF THE INVENTION

15 In order to detect the presence of a specific nucleic acid in a sample, it is generally required that large amounts of the specific sequence be present so that method such as gel electrophoresis and/or probe-specific hybridization can be performed. For qualitative purposes, amplified fragments are often analyzed by agarose gel electrophoresis. Quantitation of a transgene can be accomplished, for
20 example, through real time polymerase chain reaction (PCR), wherein an amplified product is detected as it is made. The presence of transgenes introduced by genetic modification (GM) is mainly detected through PCR reactions to detect the presence of a specific nucleic acid in a sample encoding the transgene. Such PCR reactions depend on binding of two short oligonucleotides to specific regions on template
25 DNA followed by polymerase chain reaction to amplify specific DNA fragments. Nucleic acid amplification methods, such as polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence based amplification (NASBA) and related techniques have become a central technology in genetic engineering and molecular biology. These methods enable the detection of single copy genes and/or
30 DNA fragments with a very high degree of sensitivity, due to the extreme amplification of the signal obtained. As a result, these methods are gradually

becoming critical methods in genetic diagnostics in addition to methods in research oriented environments.

In an effort to eliminate the need for sample transfers, so called "homogeneous" or "real-time" methods for the detection of PCR amplified products have been developed. One of the key advantages of real time PCR is the ability to include all necessary components for both amplification and detection in a homogeneous reaction system. While several schemes have been put forward for detecting specific nucleic acids in homogeneous solutions (see, e.g., Heller et al. European Patent Application 82303699.1, Morrison et al. (1989) Anal. Biochem., 183: 231-244, Cardullo et al. (1988) Proc. Natl. Acad. Sci. USA, 85: 8790-8794, Morrison et al. (1993) Biochem., 32: 3095-3104, and Sixon et al. (1994) Nucl. Acids Res., 22: 662-668), these methods are typically unsuitable for real-time measurements.

Important design goals for real time monitoring methods and instruments include the ability to minimize sample-to-sample variability in multi-sample thermal cycling, automation of pre- and post-reaction processing steps, high speed temperature cycling, minimization of sample volumes, real time measurement of amplification products, minimization of cross contamination, for example, due to "sample carryover" and simplification of equipment required to carry out the process.

U.S. Patent No. 5,210,015, issued to Gelfand et al., proposes fluorescence-based approaches to provide real time measurements of amplification products during PCR. Such approaches have either employed intercalating dyes (such as ethidium bromide) to indicate the amount of double-stranded DNA present, or they have employed probes containing fluorescence-quencher pairs (also referred to as the "Taq-Man" approach) where the probe is cleaved during amplification to release a fluorescent molecule whose concentration is proportional to the amount of double-stranded DNA present. During amplification, the probe is digested by the nuclease activity of a polymerase when hybridized to the target sequence to cause the fluorescent molecule to be separated from the quencher molecule, thereby causing fluorescence from the reporter molecule to appear.

Other nucleic acid hybridization probe assays utilizing fluorescence resonance energy transfer pairs include those described by Tyagi et al. in U.S. Patent No. 5,925,517, which utilizes labeled oligonucleotide probes, which we have come to refer to as "Molecular Beacons." A Molecular Beacon probe is an oligonucleotide whose end regions hybridize with one another to form a "hairpin" in the absence of a target but are separated if the central portion of the probe hybridizes to its target sequence. Non-FRET fluorescent versions of Molecular Beacon-type probes have also been described (See, *e.g.*, U. S. Patent No. 6,150,097 issued to Tyagi et al.)

These fluorescent detection approaches in general require a fluorescent compound and a second fluorophore or a quencher. Most fluorescent compounds, however, generally suffer the disadvantage that the fluorescent complexes and their binding moieties are relatively large. In addition, the presence of the fluorescer/quencher markers or the nature of the hairpin loop can alter the interaction of the labeled nucleic acid with other molecules either through chemical interactions or through steric hindrance.

Numerous other methods have been disclosed which enable detection and quantitation of nucleic acid polymerization products. A self-sustained sequence replication electrochemiluminescent nucleic acid assay is disclosed in patent application WO 94-US10732 940921 (Kenten, et al.). Hybridization protection assays have also been described. U.S. Patent No. 5,593,867, issued to Walker, et al., discloses a fluorescence polarization detection of nucleic acid amplification using fluorescently labeled oligonucleotide probes and detector-probe extension products. U.S. Patent No. 5,340,716, issued to Ullman et al., describes an assay method utilizing photoactivated chemiluminescent labels. Photoactivatable chemiluminescent matrices have also been described, as have assay methods utilizing induced luminescence as in U.S. Patent No. 6,251,581, issued to Ullman et al. U.S. Patent No. 5,017,473 issued to Wagner discloses a homogeneous chemiluminescence immunoassay using a light absorbing material.

U.S. Patent No. 6,159,693 discloses methods for detecting specific nucleic acid sequences wherein DNA or RNA probes are hybridized to target nucleic acid

sequences. Probes that are complementary to the target sequence at each base are depolymerized. The nucleic acid detection systems utilize the pyrophosphorolysis reaction catalyzed by various polymerases to produce deoxyribonucleoside triphosphates or ribonucleoside triphosphates. dNTPs are transformed to ATP by the action of NDPK. The ATP produced by these reactions is detected by luciferase or NADH based detection systems.

Methods for sequencing a target nucleic acid by detecting the sequential release of PPi's from dNTP precursors during the sequencing reaction have also been described. U.S. Patent No. 6,210,891, issued to Nyren et al., discloses a method for determining a nucleotide sequence which comprises a method wherein different deoxynucleotides or dideoxy nucleotides are either added to different aliquots of a template-primer mixture or are added successively to the same mixture and wherein the incorporation of a particular base is determined by detecting the PPi released in the sequencing reaction. U.S. Patent No. 6,258,568, issued to Nyren et al., discloses a method for determining a nucleotide sequence wherein different deoxynucleotides or dideoxy nucleotides are either added to different aliquots of a template-primer mixture or are added successively to the same mixture, wherein the incorporation of a particular base is determined by detecting the PPi released in the sequencing reaction and which further employs a nucleotide-degrading enzyme to cleave unincorporated nucleotides.

The present invention provides a modification of PPi detection during a polymerase reaction that can be used to detect the presence of a defined sequence in a sample, where PPi release is detected during the replication of a target nucleic acid.

SUMMARY OF THE INVENTION

The present invention provides new methods for the detection of a target nucleic acid in a sample and kits for carrying out methods. The method comprises replicating a new nucleic acid using the target nucleic acid in the sample as template and detecting the consumption of nucleotide triphosphate precursors as the template is replicated. The consumption of nucleotide triphosphate precursors

is detected as the release of PPi from the nucleotide triphosphate precursor that is used to synthesize the new nucleic acid from the template. The released PPi is enzymatically converted to ATP in the presence of APS and ATP sulfurylase. The ATP is then reacted with Luciferin in the presence of oxygen to generate light in the form of luminescence.

Thus, the subject matter of the present application provides methods of detecting the presence, absence, or quantity of a target nucleic acid in a sample by replicating a target nucleic acid in the sample and detecting the amount of PPi generated during the replication process. The replication process can be a linear or a logarithmic replication process. In a preferred embodiment, the replication involves a logarithmic replication (*i.e.*, amplification) of the target sequence.

In a one aspect, the method as described above is applied to allele-specific amplification. In this application, the nucleic acid template strand is a sense or antisense strand of one allele and is present in admixture. The oligonucleotide primer is complimentary to the specific allele to be amplified. As a result, the desired nucleic acid strand synthesized on the template strand is amplified preferentially over any other nucleic acid.

This invention also provides nucleic acid amplification mixtures comprising a polymerase; detection enzymes for identifying pyrophosphate release; deoxynucleotides, or optionally deoxynucleotide analogues, optionally including, in place of dATP, a dATP analogue which is capable of acting as a substrate for a polymerase but incapable of acting as a substrate for a said PPi-detection enzymes; and optionally a target specific oligonucleotide primer which hybridizes to a target DNA and facilitates polymerase-directed replication of the target sequence.

This invention also provides kits for performing nucleic acid replication or amplification or for detecting the presence absence or quantity of a nucleic acid in a sample. The kits comprise a container containing one or more enzymes, buffers, and/or any of the other reagents useful for practicing the methods of the present invention. An oligonucleotide can be immobilized as an individual dot on a two dimensional solid support, thus allowing all the amplification reactions to be processed in parallel.

DETAILED DESCRIPTION OF THE INVENTION

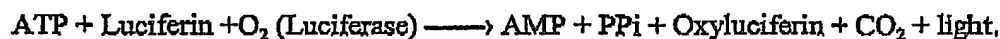
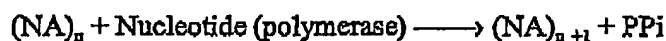
The method of the present application can be used to amplify either RNA or DNA. When used to amplify DNA, the nucleic acid polymerase is a DNA polymerase. Amplification can be linear or exponential. Linear amplification is
5 obtained when the target specific primer is the only complementary oligonucleotide used. Exponential amplification is obtained when a second oligonucleotide is present that is complementary to the target nucleic acid strand. Two primers flank the region that is targeted for amplification. RNA can be reverse transcribed into cDNA and then replicated in a linear or logarithmic fashion. When RNA is the
10 initial template, reverse transcriptase is the initial polymerase. A number of methods for enzymatic nucleic acid amplification *in vitro* have been developed and can be adapted to detect known sequence variants. These include, for example, polymerase chain reaction (Saiki et al., *Science* 230, 1350-1354 (1985)), ligase chain reaction (Landegren et al., *Science* 241, 1077-1080 (1988)), rolling circle
15 amplification (Lizardi et al., *Nature Genetics* 19, 225-232 (1998)) and other methods as known in the art.

Pyro luminescence detection is a technique based on the detection of the release of pyrophosphate (PPi) from nucleotide triphosphate precursors during template dependent nucleic acid synthesis.

20 PPi can be determined by many different methods and a number of enzymatic methods have been described in the literature (Reeves et al., (1969), *Anal. Biochem.*, 28, 282-287; Guillory et al., (1971), *Anal. Biochem.*, 39, 170-180; Johnson et al., (1968), *Anal. Biochem.*, 15, 273; Cook et al., (1978), *Anal. Biochem.* 91, 557-565; and Drake et al., (1979), *Anal. Biochem.* 94, 117-120). It is
25 preferred in the present methods to use luciferase and luciferin in combination to identify the release of pyrophosphate since the amount of light generated is substantially proportional to the amount of pyrophosphate released which, in turn, is directly proportional to the amount of base incorporated. A method for continuous monitoring of PPi release based on the enzymes ATP sulphurylase and
30 luciferase has been developed by Nyren and Lundin (*Anal. Biochem.*, 151, 504-509, 1985). The method of Nyren may be modified, for example by the use of a

more thermostable luciferase and/or ATP sulfurylase. Thus, in a preferred embodiment, luciferase and luciferin are used in combination to identify the release of pyrophosphate since the amount of light generated is substantially proportional to the amount of pyrophosphate released which, in turn, is directly proportional to the amount of base incorporated. The amount of light can readily be determined by a light sensitive device such as, e.g., a luminometer.

The preferred detection enzymes involved in the PPi detection reaction are ATP sulphurylase and Luciferase. In a cascade of enzymatic reactions, visible light is generated that is proportional to the number of incorporated nucleotides, as illustrated below, with the required enzyme for each step indicated in parentheses:



The pyro luminescent detection system can be applied to quantitatively detect the presence of a transgene in a target DNA sample. Within a time window, the amount of PPi produced is proportional to the quantity of primer: template nucleic acid for the polymerization reaction. Thus, detection can be conducted after various cycles of PCR so that the method can also be qualitative. Due to the high sensitivity and broad linear range of such a system, it is possible that just a few or even one polymerization reaction without the need for amplification cycling will yield sufficient signal for quantitation. If only one reaction is required, there is no need for a PCR machine, since the polymerization product can be directly detected with, for example a luminometer, following conversion of the PPi. The invention thus provides a method for detecting a genetically modified organism (GMO) which greatly simplifies GMO detection, cuts down operating time and lowers operating costs. Furthermore, detection of PPi with simple enzymatic reactions and luminiscence requires less skill when compared to other techniques.

of homogenous nucleic acid amplification and detection.

The present invention also contemplates the use of two or three PCR primers to amplify a specific target transgene or nucleic acid to ensure accurate results. The method of the invention may be performed in two steps, but in one
5 embodiment it is performed in a single homogenous reaction in a single tube or environment. When being performed sequentially, a polymerase reaction or a primer extension step is performed, wherein the various nucleotide(s) are incorporated, followed by a detection step, wherein the release of PPi is monitored or detected, to detect whether or not nucleotide incorporation has taken place.
10 Thus, after the polymerase reaction has taken place, samples from the polymerase reaction mix may be removed and analyzed by adding an aliquot of the sample to a reaction mixture containing the luminescence detection enzymes and reactants. Since the preferred detection and quantification is based on a luminometric reaction, this can be easily followed spectrophotometrically. The use of
15 luminometers is well known in the art and described in the literature.

Primers can be designed for any known transgene and for new genes to be introduced into the genome of a subject of interest for the purpose of modifying the genetic makeup of that subject. In one embodiment primers are designed for
20 transgenes that have been introduced into plants. Sample DNAs can be tested with these primers simultaneously and conveniently in microtiter plates. The test DNA may also be spotted onto a solid phase such as a membrane, glass slide or other solid support before primers are added and a detection or amplification reaction is carried out. Alternatively, the primers may be attached to the solid phase material. Each approach allows high throughput detection of the transgene of interest.

25 The detection methods of the present invention do not require DNA samples that are high in purity. DNAs from processed food are usually of low quality as indicated by smears of small sized genomic DNA, which represent degraded DNA templates. Even if binding of specific PCR primers to template DNA do not result in the synthesis of full length amplified products from a defined
30 template, partial replication of a target and the detection of any PPi release can still be detected. Thus, the pyro luminescent methods of detection described herein

have the same level of specificity, but without the requirement of sequence integrity between priming sites for regular PCR detection. These methods would thus be particularly useful in the detection of GMO testing in highly processed foodsamples, for example, and other samples where degradation may be an issue.

5 The detection methods may also be used in the detection of pathogens in edible food products, cosmetics, medical fluids such as blood and IV solutions, and other products. Primers specific for DNA sequences specific for known pathogens can be designed and used in the detection and amplification methods of the present application to identify contaminated lots of food product, cosmetics or medical
10 fluids.

 To carry out the method of the invention, the detection enzymes can be included in the polymerase reaction step *i.e.*, in the chain extension reaction step. Thus the detection enzymes are added to the reaction mix for the polymerase step prior to, simultaneously with or during the polymerase reaction. The reaction mix
15 for the replication step will thus include the four deoxynucleotide triphosphate precursors needed for the reaction (dATP, dTTP, dGTP and dCTP), polymerase, at least one oligonucleotide primer which can act as a template for polymerase-directed replication, and in a preferred embodiment luciferin, APS, ATP
 suphurylase and luciferase. The polymerase reaction may be initiated by addition
20 of the polymerase or the oligonucleotide primer. The detection enzymes can already be present at the time the reaction is initiated or they may be added with the reagent that initiates the reaction.

 ATP can sometimes be present in the reaction mixture during or after replication of a template, for example as an impurity or as a contaminant of dATP
25 added as dNTP precursor. Endogenous ATP may also interfere in the pyrophosphate luciferin system and give an incorrect luminescence reading. However, the endogenous ATP can be removed from a sample by contacting the sample with an immobilized enzyme such as a pyrase that converts ATP into a product which is no longer a substrate for luciferase. *See e.g.*, U.S. Patent No.
30 6,258,568. Thus, in one embodiment, replication of the target sequence is carried out using a dATP analogue which does not interfere in the enzymatic PP_i detection

reaction but which nonetheless may be normally incorporated into a growing DNA chain by a polymerase. See, *e.g.*, U.S. Patent No. 6,258,568.

5 The methods of the invention may be performed on nucleic acids attached to a solid support. Methods for detecting specific nucleic acid sequences generally involve immobilization of the target nucleic acid on a solid support such as nitrocellulose paper, cellulose paper, diazotized paper, or a nylon membrane. The solid support may also be, *e.g.*, a microtiter well or a bead. In general, any solid support may conveniently be used including any of the vast number described in the art, *e.g.*, for separation/immobilization reactions or solid phase assays, such as for example supports comprising particles, fibers or capillaries made, for example, of agarose, cellulose, alginate, Teflon or polystyrene. Magnetic particles are also useful since they can be readily isolated from a reaction mixture yet have superior reaction kinetics over many other forms of support. The solid support may carry functional groups such as hydroxyl, carboxyl, aldehyde or amino groups, or other moieties such as avidin or streptavidin, for the attachment of primers.

10 The polymerase reaction in each aliquot in the presence of the extension primer and a deoxynucleotide is carried out using a polymerase which will incorporate deoxynucleotides, *e.g.*, T7 polymerase, Klenow or Sequenase Ver. 2.0 (USB U.S.A.). However, it is known that many polymerases have a proof-reading or error checking ability which employs digestion of a growing strand which can result in a significant level of background noise when detecting PPi release. Thus, in preferred embodiments a nonproof-reading polymerase may be used or fluoride ions or nucleotide monophosphates which suppress 3' digestion by polymerase can be added to the replication reaction.

25 The reagents, including enzymes, which may be utilized in the polymerized chain reaction (PCR) are known in the art. See, for example, U.S. Patent Nos. 5,210,015; 4,683,195; 4,683,202; 4,965,188; 4,800,159; and 4,889,818, the relevant portions of which are incorporated by reference. However, preferred cycling conditions for this reaction are about 1 to 30 cycles, more preferably 5 to 20 cycles, and still more preferably 10 to 15 cycles, at alternating between temperatures of about (1) 58 °C to about 95 °C to (2) about 58 to about 95 °C.

Some of the more preferred temperature combinations being about 95° C about 58° C, about 58° C, about 74° C, and about 74° C. However, other combinations are also suitable.

5 A large variety of microorganisms are involved in the contamination of food, cosmetics, medical supplies and fluids, blood products, intravenous solutions, and the like, among other commercial products. In addition, detection of transgenes is often desirable for the detection of unwanted or contaminated bioengineered food products. The methods of the present invention can be utilized in both the detection of unwanted contaminating microorganisms or unwanted
10 contaminating bioengineered foodstuffs. The methods can also be utilized in the many steps required for the preparation, identification and propagation of new plant lines having an inserted transgene.

The nucleic acids detected with the methods of the invention can include fragments thereof, from any source in purified or unpurified form including DNA
15 and RNA, including t-RNA, m-RNA, r-RNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof. The nucleic acids can further comprise all or part of genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, humans, and the like. The nucleic
20 acids can be only a minor fraction of a complex mixture such as a biological sample and can be obtained from various biological materials by procedures well known in the art.

The oligonucleotide primers utilized in the methods of the invention can comprise in one embodiment a synthetic nucleotide that is single stranded,
25 containing a sequence at its 3'-end that is capable of hybridizing with a defined sequence of the target polynucleotide. Normally, an oligonucleotide primer has at least 80%, preferably 90%, more preferably 95%, most preferably 100%, complementarity to a defined sequence or primer binding site. The number of nucleotides in the hybridizable sequence of an oligonucleotide primer should be
30 such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridization. The oligonucleotide primer

generally will be the same as the defined sequence of the target polynucleotide, that is, generally from about 10 to 200 nucleotides in length, preferably from 20 to 50 nucleotides in length.

5 The term "nucleoside triphosphates" also includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized in a similar manner to the underivatized nucleoside triphosphates. Examples of such derivatives or analogs, by way of illustration and not limitation, are those which are biotinylated, amine modified, alkylated, and the like and also include phosphorothioate, phosphite, ring atom modified derivatives, and the like.

10 Nucleotide polymerases as used in the invention comprise a catalyst, usually an enzyme, for forming an extension of a complementary polynucleotide along a DNA or RNA template. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a polynucleotide to provide a sequence
15 complementary with the polynucleotide template. Usually, the catalysts are enzymes, such as DNA polymerases, for example, prokaryotic DNA polymerase (I, II, or III), T4 DNA polymerase, T7 DNA polymerase, Klenow fragment, reverse transcriptase, Vent DNA polymerase, Pfu DNA polymerase, Taq DNA polymerase, and the like.

20 The following Example illustrates the invention. The figures and the sequence listing referred to in the Examples are as follows:

FIG. 1: Photographs of agarose gels analyzing products of a polymerase chain reaction using the pyro luminescence detection method. Panel A shows the results following 10 cycles of PCR; Panel B shows results following 20 cycles of
25 PCT; and Panel C shows results following 30 cycles of PCR. Lane 1, DNA isolated from a maize sample containing 4% of the GMO MON810 maize; Lane 2, 149 basepair (bp) DNA fragment positive control template; Lane 3, negative control containing dATP, but no template DNA and; Lane 4, negative control containing no template DNA, and substituting the inactive dATP analog dATP α S for dATP.
30

FIG. 2. Fluorescence measurements of samples following PCR. Panel A, 10 cycles of PCR; Panel B, 20 cycles of PR; Panel C, 30 cycles of PCR. Samples

are as follows: —◆—, DNA from maize sample containing 4% of the GMO MON810; —■—, cDNA positive control; —▲—, auto fluorescence control with dATP ; —X—, auto fluorescence control with dATP α S.

FIG. 3. Results of assays of soybean and maize material containing 5% GMOs with transgenes for resistance to the herbicides glyphosate (Roundup Ready soybeans having an EPSPS gene) and glufosinate (maize having the bar gene) and insect resistance (Bt-176 maize, Bt-11 maize and MON810 maize.)

SEQ ID No. 1 – Event specific primer MG3 corresponding to the junction region of the 35S promoter and HSP intron 1 of the transgene inserted into GMO MON810 corn.

SEQ ID No. 2 – Event specific primer MG4 corresponding to the junction region of the 35S promoter and HSP intron 1 of the transgene inserted into GMO MON810 corn.

SEQ ID Nos. 3-29 – Primer pairs which may be used to detect and/or amplify known transgenes in plants.

EXAMPLE 1

Detection of the Event Specific Target Nucleic Acid of MON810 Maize

Reference samples of maize containing the transgenic GMO corn line MON810 were obtained from Sigma Aldrich Chemical Company. Genomic DNA was isolated from the maize sample using the High Pure GMO sample preparation kit available from Roche Diagnostics Corporation of Indianapolis, IN. A pair of event specific primers, MG3 : 5'– agt atc ctt cgo aag acc ctt oct c– 3' (SEQ ID NO. 1) and MG4 :5'– gca ttc aga gaa acg tgg cag taa c– 3' (SEQ ID NO. 2) were used to amplify a 149 basepair fragment of the transgene corresponding to the region of the junction of 35S promoter and HSP intron 1 of MON810 maize.

PCR REACTION: PCR was performed using the PTC-100 thermal cycler (available from MJ Research, Inc). The reaction was carried out in a total volume of 30 μ l and contained 0.20 μ M of each of the primers MG3 and MG4, 0.25mM of each of the four dNTPs (dATP, dCTP, dGTP, dTTP), 1.5mM MgCl₂, 1.5 units of Taq DNA polymerase and 1 μ l (10ng-100ng) template genomic DNA. The Taq DNA polymerase was from Qiagen.

The thermal cycler program consisted of the following steps: a denaturation step of 4 minutes at 94° C; cycling steps consisting of a 45 second incubation at 94° C, a 30 second incubation at 60° C, and a 45 second incubation at 72° C; and an extension step of 7 minutes at 72° C. The reactions were conducted in three sets and allowed to run for 10, 20 and 30 cycles respectively.

FLUORESCENCE DETECTION: Autofluorescence was carried out by pyro luminescence, a technique that detects the release of pyrophosphate (PPi) from nucleotide triphosphates during template dependent nucleic acid synthesis by means of a Luciferase-Luciferin-based reaction. The reaction was carried out in a buffer containing 0.1M Tris-acetate, 2mM EDTA, 10mM Magnesium acetate, 0.1% bovine serum albumin, 1mM dithiothreitol, 0.4 mg/ml polyvinylpyrrolidone, 100µg/ml Luciferin, 0.3 units/ml ATP sulfurylase, 5µM Adenosine 5'-phosphosulfate and 20ng Luciferase. (The Luciferin, Luciferase and ATP sulfurylase were obtained from Sigma Aldrich Chemical Company.) A 5µl sample of the PCR reaction products was mixed with 100 µl of fresh autofluorescence detection buffer immediately before fluorescence readings were taken. The readings were taken at 15 second-intervals for 2 minutes in a VersaFluor Fluorometer (available from Bio-Rad Laboratories). Deionized water was used as the blank.

RESULTS: Event specific target DNA in genomic DNA isolated from a reference sample which contains 4% of the GMO MON810 maize was detected and amplified by PCR. The products of the PCR were analyzed by agarose gel electrophoresis, as shown in Fig. 1 and by fluorescence detection, as shown in Fig. 2. Ten µl of the PCR products were separated on a 2% agarose gel in 1X TAE buffer. (The gel also contained a 100-bp molecular marker available from New England Biolabs.)

A specific 149 bp DNA fragment of the transgene inserted into MON810 corn was amplified (data not shown) using the two event specific primers MG3 (SEQ ID NO:1) and MG4 (SEQ ID NO:2). The 149 bp fragment was gel purified and used as the positive control template for the PCR data presented in Fig. 1. The PCR amplification product of this fragment is shown in lanes 2 of panels A, B and C of Fig. 1. Corresponding autofluorescence readings are presented in Fig. 2 (—■—).

The PCR product of the reaction having maize genomic DNA is presented in Lane 1 of Fig. 1 and indicated by —◆— in Fig. 2. Negative control reactions having no template DNA were also carried to determine if the presence of dATP in PCR reaction creates a background fluorescence too high to allow detection of amplified DNA product. The results of this reaction are shown in lane 3 of panels A, B and C of Fig. 1 and the fluorescence measurements of the corresponding panels A, B and C of Figure 2. (—▲—). A second negative control having no template DNA, and including the inactive dATP analog dATP α S at a final concentration of 0.25 mM rather than dATP, was done to evaluate the background fluorescence levels produced by the dATP in the PCR reactions.

The results demonstrate that the autofluorescence level is high for the initial 15 seconds of the reaction, but it gradually stabilizes between 30 seconds and 2 minutes to provide a workable window during which reliable autofluorescence measurements can be made.

A 149 bp DNA fragment is detectable on the agarose gel for the positive control (Panel A, lane 2) after ten cycles of PCR at an autofluorescence level much higher than two negative controls having no template DNA. Moreover, the two negative controls with dATP and dATP α S have comparable levels of autofluorescence, suggesting that the carryover of dATP from the PCR reaction does not seriously increase autofluorescence background. The use of dATP α S in the reaction does not substantially effect the levels of autofluorescence obtained.

In the sample containing the genomic DNA from maize material comprising 4% of the GMO MON810, the specific 149 bp fragment amplified by primers MG3 and MG4 only becomes detectable after 30 cycles of PCR. Background levels of autofluorescence, in contrast, are detectable after only 10 cycles of PCR. This result suggests that better sensitivities will be obtained by carrying out PCR for multiple cycles as one would do for quantitative PCR, than by carrying out fewer cycles as one would do for qualitative PCR.

The present results demonstrate that detection of pyrophosphate levels by Luciferin autofluorescence in a sample following a PCR reaction can be used to detect the presence of the transgenes of GMOs. In this method, the carryover of a small amount of dATP from the PCR reaction does not create background levels of

fluorescence that interfere with detection and measurement of the specific nucleic acid of the transgene that is being detected.

EXAMPLE 2

Plant material obtained from Sigma Aldrich Chemical Company containing 5% Roundup Ready Soybeans, 5% Bt 176 maize or 5% Bt-11 maize have been analyzed for the presence of the specific transgenes which provide the insect resistance and herbicide resistance traits to commercial plant lines using the methods described in Example 1. The results of these detection and amplification experiments are presented in Fig. 3. The sequences of the primer pairs used in the assays are given in Table 1 below. The results of these experiments demonstrate the general applicability of the methods of the present application to the identification of a variety of different target nucleic acid sequences in a sample to be analyzed for small amounts of those target sequences.

TABLE 1

Primer	Sequence	
P35S1-5/2-3	5' att gat gtg ata tct cca ctg acg t 3'	SEQ ID NO. 3
	5' cct ctc caa atg aaa tga act tcc t 3'	SEQ ID NO. 4
P35S-cf-3/4	5' cca cgt ctt caa agc aag tgg 3'	SEQ ID NO. 5
	5' tcc tct cca aat gaa atg aac ttc c 3'	SEQ ID NO. 6
P35S1-5'/nos-3	5' att gat gtg ata tct cca ctg acg t 3'	SEQ ID NO. 7
	5' tta tcc tag ttt gcg cgc ta 3'	SEQ ID NO. 8
Mg 1/2	5' tat ctc cao tga cgt aag gga tga c 3'	SEQ ID NO. 9
	5' tgc cct ata aca cca aca tgt gct t 3'	SEQ ID NO. 10
Mg 3/4	5' agt atc ctt cgo aag acc ctt cct c 3'	SEQ ID NO. 1
	5' gca ttc aga gaa acg tgg cag taa c 3'	SEQ ID NO. 2
Cd01/cr02	5' cgg atg gtc ctt atg caa ttt tgt c 3'	SEQ ID NO. 11
	5' ctc tgg gcg tag att tgg tac a 3'	SEQ ID NO. 12

	Primer	Sequence	
	Pe01/cr02	5' aga ttc ttc act cag atg cag cct a 3' 5' ctc tcg gcg tag att tgg tac a 3'	SEQ ID NO. 13 SEQ ID NO. 12
	Cry1a2-5' t35S3-3'	5' tgg aca aca acc caa aca tca a 3' 5' tgg att ttg gtt tta gga att aga aa 3'	SEQ ID NO. 14 SEQ ID NO. 15
	Cry1a4-5'/3-3'	5' gga caa caa cca caa cat caa c 3' 5' cga tgg ggg tgt aac cgg t 3'	SEQ ID NO. 16 SEQ ID NO. 17
	Cry1a4-5'/4-3'	5' gga caa caa cca caa cat caa c 3' 5' goa cga act cgc taa gca g 3'	SEQ ID NO. 16 SEQ ID NO. 18
5	Cry1a1/2	5' egg ccc cga gtt cac ctt 3' 5' ctg ctg ggg atg atg ttg ttg 3'	SEQ ID NO. 19 SEQ ID NO. 20
	Cm01/cr02	5' cac tac aaa tgc cat cat tgc gat a 3' 5' ctc tcg gcg tag att tgg tac a 3'	SEQ ID NO. 21 SEQ ID NO. 12
	Cm01/pa01	5' cac tac aaa tgc cat cat tgc gat a 3' 5' aga tca tca atc cac tct tgt ggt g 3'	SEQ ID NO. 21 SEQ ID NO. 22
	Cm01/bar1-5'	5' cac tac aaa tgc cat cat tgc gat a 3' 5' gat agc got ccc gca gac 3'	SEQ ID NO. 21 SEQ ID NO. 23
10	Adh1 1-5'/nos-ter2-3'	5' gca ctg aat ttg tga acc c 3' 5' ota tat ttt gtt ttc tat cgc 3'	SEQ ID NO. 24 SEQ ID NO. 25
	Bar3-5'/1-3'	5' cat cgt caa cca cta cat cga ga 3' 5' gat agc gct ccc goa gac 3'	SEQ ID NO. 26 SEQ ID NO. 27
	Bar2-5'/2-3'	5' act ggg otc cac gct cta ca 3' 5' aaa coc acg tca tgc cag tto 3'	SEQ ID NO. 28 SEQ ID NO. 29

CLAIMS

1. A method of identifying the presence of a target nucleic acid in a sample wherein the target nucleic acid is replicated and said replication of the target is
5 detected as the consumption of a deoxynucleotide triphosphate precursor, the method comprising:
adding an oligonucleotide primer which hybridizes to the sample target nucleic acid to the sample;
subjecting the sample DNA and primer to a polymerase reaction in the
10 presence of a mixture of all dNTP's required for replication of the target nucleic acid whereby the deoxynucleotides will become incorporated and release pyrophosphate (PPi) proportional to the length of the DNA extension product; and
detecting any release of PPi enzymatically;
whereby any release of PPi is indicative of incorporation of deoxynucleotide or
15 dideoxynucleotide and the presence of the target DNA.
2. A method as in claim 1, wherein the target nucleic acid is replicated in a reaction selected from the group consisting of a polymerase extension reaction, a polymerase chain reaction(PCR), a ligase chain reaction (LCR), a rolling circle
20 replication reaction (RCR) and a nucleic acid sequence based amplification reaction (NASBA).
3. A method as in claim 2 wherein the target is replicated in a polymerase chain reaction.
- 25
4. A method as claimed in claim 1, wherein the release of PPi is detected by means of a Luciferase-Luciferin-based reaction.
5. A method as claimed in claim 1, wherein PPi release is detected using ATP
30 sulfurylase and luciferase.
6. A method as claimed in claim 1, wherein the PPi detection enzymes are

included in the polymerase reaction step and the polymerase reaction and PPi release detection steps are performed substantially simultaneously.

5 7. A method as claimed in claim 1, further comprising adding a dATP analogue which is capable of acting as a substrate for a polymerase, but incapable of acting as a substrate for a PPi detection enzyme.

10 8. A method as claimed in claim 7, wherein the dATP analogue is deoxyadenosine .alpha. thiotriphosphate

9. A method as claimed in claim 1, wherein the sample DNA or oligonucleotide primer is immobilized or provided with means for attachment to a solid support.

15 10. A method as claimed in claim 1, wherein an exonuclease deficient high fidelity polymerase is used.

11. A method as in claim 1 wherein a heat resistant polymerase is used.

20 12. A method as claimed in claim 1, wherein the sample DNA is first amplified.

13. A method as claimed in claim 1, for use with a multiplicity of sample DNA sequences, wherein said DNA sequences are arranged in assay format on a solid surface.

25 14. The method according to claim 1 wherein said nucleic acid sample is obtained from a biological sample.

15. The method according to claim 1 wherein said target nucleic acid is a microbial or viral nucleic acid.

30 16. The method according to claim 15 wherein said target nucleic acid target is a viral nucleic acid.

17. The method according to claim 1 wherein said nucleic acid sample is obtained from a food source.
- 5 18. The method according to claim 17 wherein said food source is a plant.
19. The method according to claim 1 wherein said target nucleic acid contains a nucleic acid sequence non-native to the sample.
- 10 20. A kit for use in a method as defined in claim 1, comprising:
a polymerase;
detection enzyme means for identifying PPi release;
dNTP's, or optionally deoxynucleotide analogues, optionally including, in
place of dATP, a dATP analogue which is capable of acting as a substrate for a
15 polymerase but incapable of acting as a substrate for a said PPi-detection enzyme; and
optionally a target specific primer which hybridizes to the target DNA and is
recognized as a primer by a polymerase, wherein the polymerase replicates the target
DNA.
- 20 21. A kit as claimed in claim 20, wherein the detection enzyme means comprise a
Luciferase-Luciferin-based enzymatic reaction.
22. A kit as claimed in claim 21, wherein the detection enzyme means comprise
ATP sulfurylase and luciferase.

25

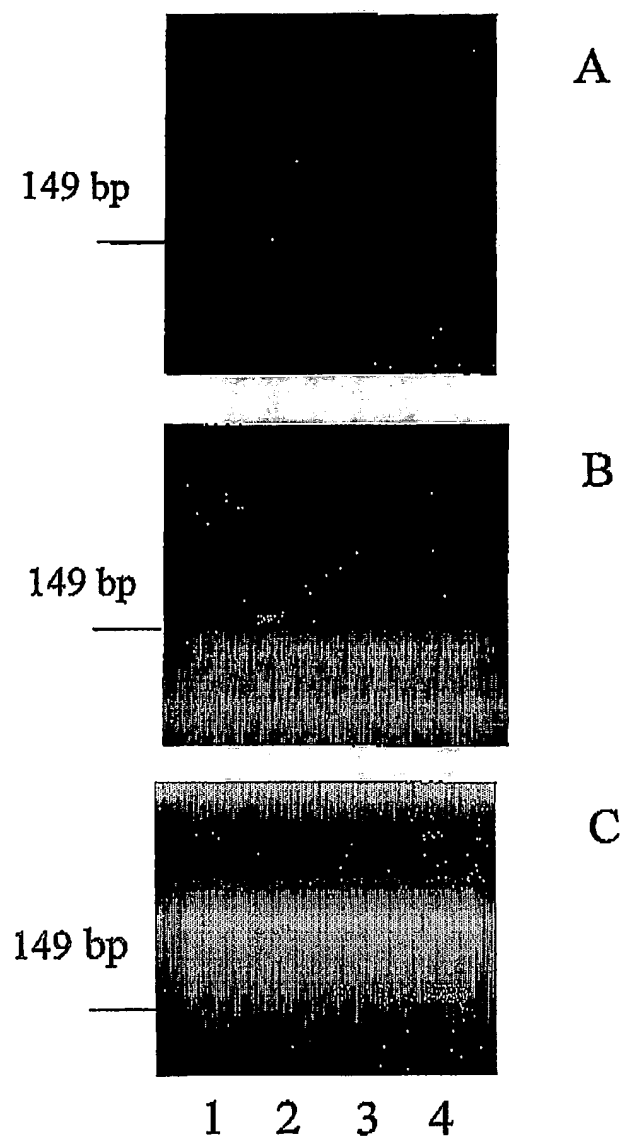


Fig. 1

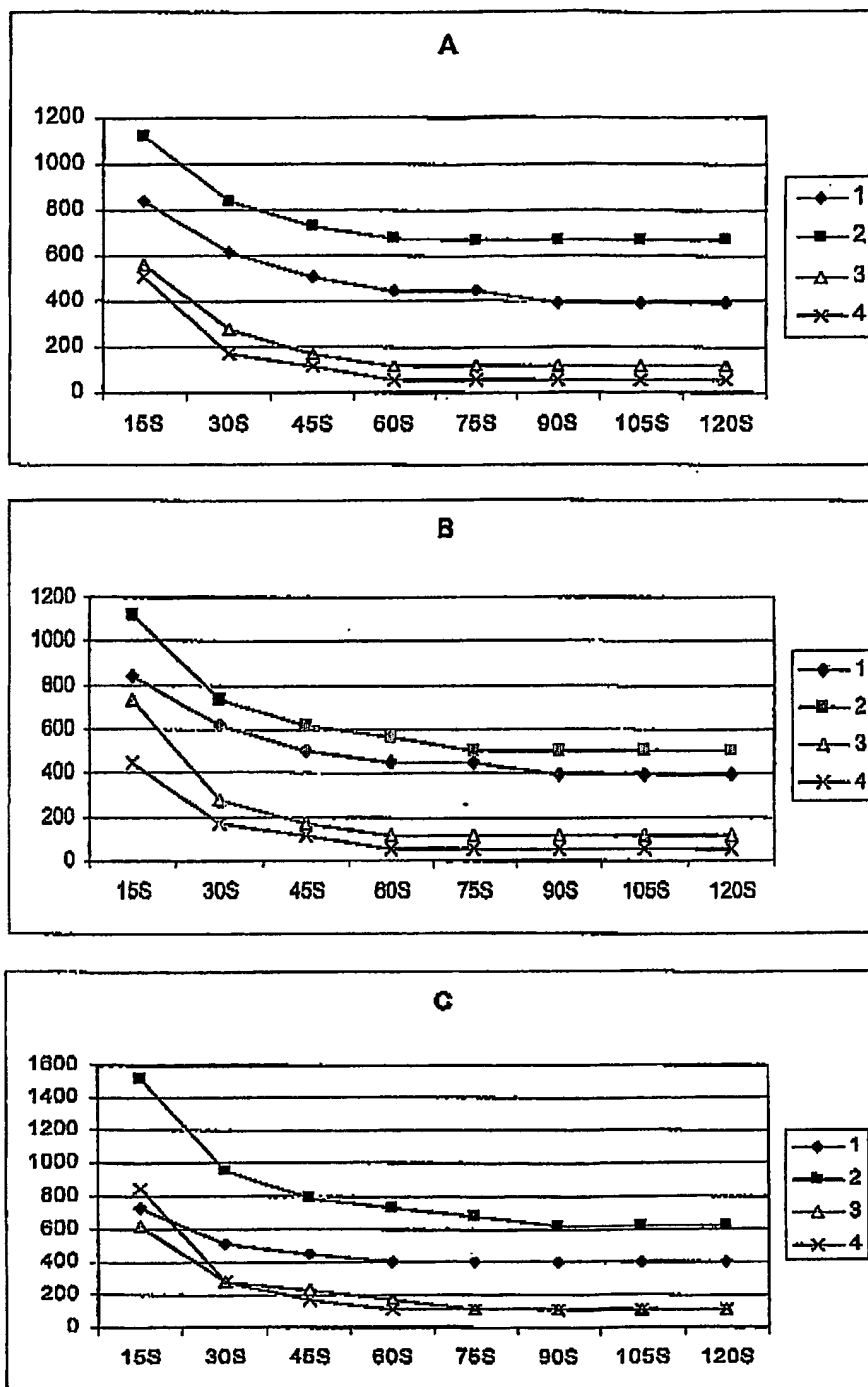


Fig 2

Primer (sense/ antisense)	Amplicon (size)	IRMM-410S Roundup Ready soya bean 5%	IRMM-411R BT-176 maize 5%	IRMM-412R BT-11 maize 5%	IRMM-413R Mon 810
P35a1-5'/2-3'	P35s(101bp)				
P35s-cf-3/4	P35s(123bp)	ND			
P35s1-5'/Nos-3	CMV/Nos-ter(1.1kb)	-	ND		-
Mg1/2	P35s/HSP intron 1(401bp)	-	ND	-	
Mg3/4	P35s/HSP intron 1(149bp)	-	ND		
CD01/CR02	CDPK	ND		ND	ND
PE01/CR02	Pro/CryIA(b)(900bp)	ND		ND	ND
	PEPC Pro/ CryIA(b) (900bp)	ND		ND	ND
Cry1A2- 5'/T35s3-3'	CryIA(b)/CMV(2100bp)	ND		ND	ND
Cry1A4-5'/3-3'	CryIA(b)(107bp)	-			-
Cry1A4-5'/4-3'	CryIA(b)(152bp)	-			
Cry1A1/2	CryIA(b)(420bp)	-	ND		
CM01/CR02	CMV/ CryIA(b)(1.2kb)	-	ND		-
CM01/PA01	CMV/Pat(700bp)	-	ND		-
CM01/ Bar1-5'	CMV/Bar(600bp)	ND		ND	ND
Adh1-5'/Nos- ter2-3'	Adh1-STVS2/Nos- ter(2.1kb)	-	ND		-
Bar2-5'/2-3'	Bar(186bp)	ND		ND	ND
Bar3-5'/1-3'	Bar(104bp)	ND		ND	ND

-.: negative; +: positive band that can also be detected by pyroluminescence; ND: not done

FIG. 3

SEQUENCE LISTING

<110> Temasek Life Sciences Laboratory

<120> DETECTION OF TRANSGENES OF GENETICALLY MODIFIED ORGANISMS
USING PYRO LUMINESCENCE

<130> 2577-158

<160> 29

<170> PatentIn version 3.3

<210> 1

<211> 25

<212> DNA

<213> Primer

<400> 1
agtatccttc gcaagaccct tcctc
25

<210> 2

<211> 25

<212> DNA

<213> Primer

<400> 2
gcattcagag aaacgtggca gtaac
25

<210> 3

<211> 25

<212> DNA

<213> Primer

<400> 3
attgatgtga tatctccact gacgt
25

<210> 4

<211> 25

<212> DNA

<213> Primer

<400> 4
cctctccaaa tgaaatgaac ttcct
25

<210> 5
<211> 21
<212> DNA
<213> Primer

<400> 5
ccacgtcttc aaagcaagtg g
21

<210> 6
<211> 25
<212> DNA
<213> Primer

<400> 6
tcctotccaa atgaaatgaa ctccc
25

<210> 7
<211> 25
<212> DNA
<213> Primer

<400> 7
attgatgtga tatctccact gacgt
25

<210> 8
<211> 20
<212> DNA
<213> Primer

<400> 8
ttatcctagt ttgcgcgcta
20

<210> 9
<211> 25
<212> DNA
<213> Primer

<400> 9
tatctccact gacgtaaggg atgac
25

<210> 10

<211> 25
<212> DNA

<213> Primer

<400> 10
tgccctataa caccaacatg tgctt
25

<210> 11
<211> 25
<212> DNA
<213> Primer

<400> 11
cggatgggtcc ttatgcaatt ttgtc
25

<210> 12
<211> 22
<212> DNA
<213> Primer

<400> 12
ctctcggcgt agatttggtg ca
22

<210> 13
<211> 25
<212> DNA
<213> Primer

<400> 13
agattcttca ctccgatgca gccta
25

<210> 14
<211> 22
<212> DNA
<213> Primer

<400> 14
tggacaacaa cccaaacatc aa
22

<210> 15
<211> 26

<212> DNA
<213> Primer

<400> 15

tggatttttg ttttaggaat tagaaa
26

<210> 16
<211> 22
<212> DNA
<213> Primer

<400> 16
ggacaacaac cacaacatca ac
22

<210> 17
<211> 19
<212> DNA
<213> Primer

<400> 17
cgatgggggt gtaaccggt
19

<210> 18
<211> 19
<212> DNA
<213> Primer

<400> 18
gcacgaactc gctaagcag
19

<210> 19
<211> 18
<212> DNA
<213> Primer

<400> 19
cggccccgag ttcacatt
18

<210> 20
<211> 23
<212> DNA

<213> Primer

<400> 20

ctgctgggga tgatgttggt cmg
23

<210> 21

<211> 25

<212> DNA

<213> Primer

<400> 21

cactacaaat gccatcattg cgata
25

<210> 22

<211> 25

<212> DNA

<213> Primer

<400> 22

agatcatcaa tccactottg tgggtg
25

<210> 23

<211> 18

<212> DNA

<213> Primer

<400> 23

gatagcgctc ccgcagac
18

<210> 24

<211> 19

<212> DNA

<213> Primer

<400> 24

gcactgaatt tgtgaaccc
19

<210> 25

<211> 21

<212> DNA

<213> Primer

<400> 25
ctatatatttg ttttctatcg c
21

<210> 26
<211> 23
<212> DNA

<213> Primer

<400> 26
catcgtaac cactacatcg aga
23

<210> 27
<211> 18
<212> DNA
<213> Primer

<400> 27
gatagcgctc ccgcagac
18

<210> 28
<211> 20
<212> DNA
<213> Primer

<400> 28
actgggctcc acgctctaca
20

<210> 29
<211> 21
<212> DNA
<213> Primer

<400> 29
aaacccacgt catgccagtt c
21

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2004/000093

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl. 7: C12Q 1/68, 1/66		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) AS BELOW		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AS BELOW		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPIDS, CAPLUS, MEDLINE ; Keywords ; pyrophosphate, nucleic, DNA, polymerase, detect, luciferase.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 1998/013523, (PYROSEQUENCING AB) 2 April 1998. (See particularly p. 3 line 7 - p. 6, line 20 and claims 1, 10)	1 - 22
X	WO, A, 1998/028440, (PYROSEQUENCING AB) 2 July 1998 (See particularly p. 3 line 5 - p. 6, line 12 and claims 1, 16 and 17)	1 - 22
X	US, A, 4971903, (EDWARD HYMAN) 20 November 1990 (see particularly the Example and claims 1 - 4)	1 - 22
X	EP, A, 630974 (EASTMAN KODAK COMPANY) 28 December 1994 (see particularly Example and claims 1 - 10)	1 - 22
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 24 May 2004		Date of mailing of the international search report - 3 JUN 2004
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer ALISTAIR BESTOW Telephone No : (02) 6283 2450

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2004/000093

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A2, 2002/064830 (CAMBRIDGE UNIVERSITY TECHNICAL SERVICES) 22 August 2002. (see particularly Example 2 and claims 1 - 17)	1 - 22
X	WO, A, 2000/040750 (AMERSHAM PHARMACIA BIOTECH AB) 13 July 2000, (see particularly p. 2 line 32 - p. 4 line 2, Example 1 and claims 1 - 7)	1 - 22
X	WO, A, 1998/055653 (NEXSTAR PHARMACEUTICALS, INC.) 10 December 1998. (see particularly p. 7 line 14 - p. 9 line 24, Examples and claims 1, 23 - 25, 34 - 39)	1 - 22
X	WO, A, 1992/016654 (UNIVERSITE DE REIMS CHAMPAGNE-ARDENNE) 1 October 1992 (see examples and claims)	1 - 22
P,X	US ,A1 2003/0082583 (ARJANG HASSIBI ET. AL.) 1 May 2003.(see particularly claims 1 - 35)	1 - 19
X	RONAGHI, M., et. al. 'Real-Time DNA Sequencing Using Detection of Pyrophosphate Release' Analytical Biochemistry (1996) 242:84-9 (see particularly Abstract and Figure 1)	1 - 22
X	NYRÉN, P., et. al. 'Solid Phase DNA Minisequencing by an Enzymatic Luminometric Inorganic Pyrophosphate Detection Assay' Analytical Biochemistry (1993) 208:171-5. (See particularly Abstract and Figure 1)	1 - 22
X	ALDERBORN, A., et. al. 'Determination of Single-Nucleotide Polymorphisms by Real-time Pyrophosphate DNA Sequencing' Genome Research (2000) 10:1249-58 (see particularly Methods)	1 - 22
X	RONAGHI, m., et. al. 'A Sequencing Method Based on Real-Time Pyrophosphate' Science (1998) 281:363-5 (see particularly Fig. 1)	1 - 22
X	IMAMURA, O. et. al. 'Simple and Rapid Bioluminescent Detection for Allele Specific PCR of <i>E. COLI</i> O 157:H7' Bioluminescence & Chemiluminescence: Progress & Current Applications, [Proceedings of the Symposium on Bioluminescence and Chemiluminescence], 12th, Cambridge, United Kingdom, Apr. 5-9, 2002 (2002), 395-398.	1 - 22
X	NYRÉN, P., et. al. 'Detection of Single-Base Changes Using a Bioluminometric Primer Extension Assay' Analytical Biochemistry (1997) 244:367-73. (see particularly Abstract and Fig. 1)	1 - 22
X	NYGREN, M., et. al. 'Quantification of HIV-1 Using Multiple Quantitative Polymerase Chain Reaction Standards and Bioluminometric Detection' Analytical Biochemistry (2001) 288:28-38. (see particularly	1 - 22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2004/000093

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: **20 - 22 (partially)**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The claimed kits have been searched only to the extent that they are being used in the method of the invention.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/SG2004/000093

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	9813523	AU	43918/97	EP	0932700	US	6210891
WO	9828440	AU	53311/98	EP	0946752	US	6258568
		US	2003104372	WO	02068684		
US	4971903	AU	33548/89	WO	8909283		
EP	0630974	JP	7059600				
WO	02064830	CA	2440698	EP	1360330		
WO	0040750	AU	19838/00	AU	35370/01	AU	44105/01
		CA	2357775	CA	2395159	EP	1141401
		EP	1239962	EP	1242186	US	2003143114
		US	2003146155	WO	0146465	WO	0147638
WO	9855653	AU	78136/98				
WO	9216654	AU	16460/92	FR	2674254		
US	2003082583	EP	1415004	WO	03087388		
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☒ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.